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MINIREVIEW

Metal-Responsive Transcription Factors That Regulate Iron, Zinc, and Copper Homeostasis in Eukaryotic Cells

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Iron, copper, and zinc are all essential nutrients. The electron transfer properties of iron and copper are fundamental to processes such as respiration and photosynthesis. Zinc forms the catalytic center in numerous enzymes and has an important structural role in a wide range of proteins. However, all these metals can be toxic if their levels and distribution are not carefully regulated, as their inappropriate binding may compromise cellular function. The uncontrolled redox activity of iron and copper can also lead to the generation of damaging oxygen radicals. Therefore, organisms maintain cytoplasmic metal concentrations at a nontoxic level that is sufficient for growth. A variety of homeostatic mechanisms have been identified, which include the control of translation and RNA stability by iron-regulatory proteins and the metal-dependent trafficking or degradation of metal transporters (39, 109, 138). This review focuses on the role that metal-responsive transcription factors have in regulating trace metal metabolism. These factors are able to sense changes in metal concentrations and coordinate the expression of genes that are involved in the acquisition, distribution, sequestration, and use of metals. Consequently, the ability to mediate metal-responsive gene expression is an important aspect of metal homeostasis in those organisms that contain these factors.

IRON

Iron is extremely insoluble in the presence of oxygen at physiological pH. Organisms that live in an oxygen environment have therefore evolved specific mechanisms to acquire what would otherwise be an unavailable nutrient. These systems of iron acquisition in many fungi are regulated at the transcriptional level by iron availability. The proteins that mediate this control are, to date, the only known iron-responsive transcription factors within eukaryotes. In the budding yeast *Saccharomyces cerevisiae*, genes that are involved in the compartmentalization and use of iron are regulated in a similar way to those genes that are involved in iron acquisition. This global regulation of iron metabolism may be established to be the norm in other eukaryotic microorganisms.

The iron metabolism of *S. cerevisiae* has been the most intensively studied of all the fungi (reviewed in references 83, 114, and 146). This organism can grow in both aerobic and anaerobic environments and can utilize a variety of carbon sources by using both fermentative and respiratory metabolism. This range of growth conditions influences iron availability and the cell requirements for iron. Under anaerobic conditions, iron is in the ferrous form and therefore more readily available. Conversely, cells that are respiring require additional iron for the various iron-containing proteins of the mitochondrial respiratory chain at a time when iron is less soluble. Therefore, mutations that are detrimental to iron metabolism often result in a more severe phenotype when this yeast grows by using a respiratory carbon source.

S. cerevisiae contains a variety of genes that are transcriptionally induced in response to low iron and which encode proteins that are involved in iron acquisition at the cell surface (Table 1; Fig. 1A). Free iron is taken into the cell by both high- and low-affinity transport systems (Fet3, Ftr1, and Fet4) (36, 136). The high-affinity complex contains a ferroxidase (Fet3) that requires copper as a cofactor. Consequently, genes that are involved in the trafficking and transport of copper to this protein (*ATX1* and *CCC2*) are also regulated at the transcriptional level by iron (93, 156, 160). High-affinity iron uptake is therefore compromised when cells experience low copper levels. A cell surface ferric reductase activity is also required for high-affinity iron uptake (33, 34). The majority of this activity is provided by two flavocytochromes (Fre1 and Fre2) that reduce ferric iron to provide ferrous iron as a substrate for the high-affinity transport system (48, 49). *S. cerevisiae* can also acquire iron through siderophores, which are low-molecular-weight organic molecules that specifically chelate iron. *S. cerevisiae* does not synthesize its own siderophores, but it is able to utilize those that are produced by other microorganisms (114). A family of transporters (Arn1 to Arn4) that cycle between the cell surface and an endosomal compartment mediate siderophore uptake (162). As an alternative to siderophore uptake, the siderophore iron can be reduced by the cell surface reductases (Fre1 to Fre4) to provide ferrous iron as a substrate for the Fet3/Ftr1 high-affinity uptake system (162). A group of mannoproteins (Fit1 to Fit3) facilitates siderophore iron uptake by sequestering this iron chelate within the cell wall (117). In addition to those genes that are involved in cell surface iron acquisition, a number of genes involved in other aspects of iron metabolism are transcriptionally induced under low-iron conditions (Table 1). These include genes that encode vacuolar transport systems (Fet5, Fth1, and Smf3), a mitochondrial

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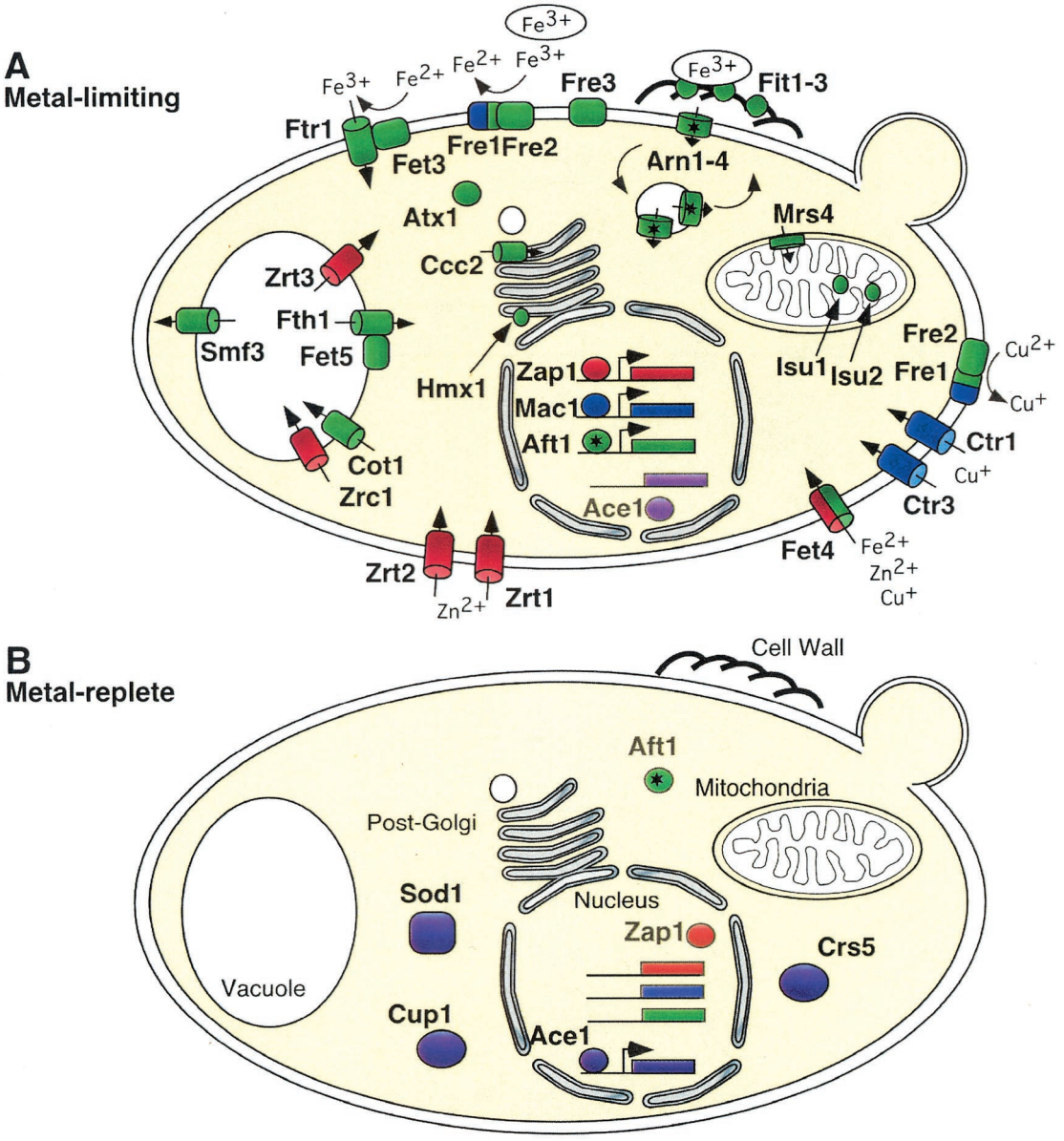

FIG. 1. Protein products of metalloregulated genes involved in metal homeostasis in *S. cerevisiae*. Products of genes that are activated under metal-limiting conditions (A) and metal-replete conditions (B) by Aft1 (green), Mac1 (blue), Zap1 (red), and Ace1 (purple) are shown. Iron that is bound to siderophores has been circled, and stars indicate proteins that undergo iron-dependent cellular trafficking. The metal ion specificities of proteins required for metal uptake are indicated. See the text for further details of the functional roles of each protein.

transporter (Mrs4), and proteins involved in the biosynthesis of iron-sulfur clusters (Isu1 and Isu2) (43, 47, 115, 116, 129, 145).

Iron-dependent gene regulation in *S. cerevisiae* is mediated by two transcription factors. Aft1 and Aft2 (for "activator of ferrous transport") activate gene expression when iron is scarce. Consequently, strains that lack both these factors exhibit reduced expression of the iron regulon (14, 24, 124, 154, 156). The genes that code for these factors are thought to have arisen from a genome duplication event (130). As with many other paralogous genes within *S. cerevisiae*, *AFT1* and *AFT2* code for proteins that have significant regions of identity and overlapping functions. The DNA-binding domain of each protein is in a highly conserved N-terminal region, and a conserved cysteine-to-phenylalanine mutation in both proteins generates a factor that activates the high expression of the iron regulon irrespective of iron concentrations (124, 154). There are clear phenotypic differences in strains that separately lack Aft1 and Aft2. An *aft1* null strain exhibits low ferrous iron uptake and grows poorly under low-iron conditions or on a respiratory carbon source (24, 154). No phenotype has been attributed to an *aft2* null strain. An *aft1 aft2* double null strain is, however, more sensitive to low-iron growth than a single *aft1* null strain, which is consistent with the functional similarity of these factors (14, 124). The partial redundancy of these factors

allows Aft2 to complement an *aft1* null strain when it is overexpressed from a plasmid (124).

The properties of Aft1 and Aft2 that distinguish them from each other have not been fully identified. Both factors mediate gene regulation via an iron-responsive element that contains the core sequence 5′-CACCC-3′ (125, 156). It is likely that sequences adjacent to this element influence the ability of each factor to mediate regulation via a particular iron-responsive element (125). The differential regulation of individual genes by Aft1 and Aft2 results in each factor generating a distinct global transcriptional profile (125). Aft1 autoregulation, which is consistent with the in vivo binding of Aft1 to its own promoter, may influence Aft1 control of the iron regulon (89). Critical to the function of Aft1 is its ability to shuttle between the nucleus and cytoplasm in response to iron levels. Nuclear export is dependent on a leucine rich N-terminal nuclear export signal (NES), mutation of which results in the nuclear retention of Aft1 and constitutive expression of Aft1-regulated genes (157). Aft1 nuclear import is mediated by a direct interaction with the nuclear import factor Pse1 (144). Aft1 contains two distinct basic nuclear localization signals, which together are sufficient and necessary to direct Aft1 to the nucleus (144).

Aft1 function is regulated in response to glucose levels independently of iron. Certain genes in the iron regulon are induced immediately following entry into the diauxic shift when cells adapt from fermentative to respiratory metabolism. This control is dependent on both the global regulatory complex Snf1/Snf4 and on Aft1 (64). The iron regulon also responds to glucose levels via the cyclic AMP-dependent protein kinase A. *TPK2* encodes a protein kinase A catalytic subunit, and a *tpk2* null strain shows derepressed expression of the iron regulon (123). Tpk2-dependent and Snf1/Snf4-dependent regulation of the iron regulon is consistent with an increased requirement for iron during respiratory metabolism (64, 123). Direct phosphorylation of Aft1 that is independent of Snf1 and cyclic AMP levels occurs when cells undergo a transient or permanent cell cycle arrest. Conditions that result in this modification of Aft1 include the change or removal of a carbon source, traversal of the diauxic shift, and the shifting of temperature-sensitive *cdc*28 or *cdc*25 mutants to a nonpermissive temperature (24, 64). The functional consequences of this cell cycle-dependent phosphorylation of Aft1 are not clear.

The mechanisms(s) by which the Aft1 and Aft2 factors sense iron are not fully understood. Nucleocytoplasmic shuttling of Aft1 in response to iron is fundamental to the ability of a cell to sense iron. However, the signal that shifts the equilibrium of Aft1 localization that results in nuclear translocation is not known. It is also not clear if that signal acts at the level of Aft1 nuclear export or import. The phosphorylation of Aft1 and the effect of the various phosphorylation pathways on the iron regulon described above are not involved in iron sensing per se. These regulatory pathways integrate Aft1 function with other aspects of cellular metabolism such as carbon source utilization. However, phosphorylation by an unknown signaling pathway could be the triggering event for Aft1 nuclear localization. Alternatively, Aft1 may bind iron directly and the loss of iron binding could initiate the Aft1 response. Direct metal binding of iron as a signal of iron status has been demonstrated with various prokaryotic iron-responsive transcription factors (reviewed in reference 6). Consistent with this hypothesis, the iron regulon in *S. cerevisiae* is sensitive to the intracellular chelation of iron and mutants that accumulate iron in the mitochondria exhibit enhanced expression of the iron regulon (44, 63). In addition, iron-responsive gene regulation is significantly compromised in cells that are unable to synthesize heme (28). The Fe(II)/Fe(III) redox equlibrium in the cell may also influence iron metabolism (63). This is supported by the phenotype of a *sod1* null strain that lacks superoxide dismutase activity. This mutant is sensitive to oxidative stress and has higher than wild-type levels of Fe(III) with a concurrent increase in the expression of an Aft1/2-regulated gene (35, 134).

Iron-regulated gene expression in fungi other than *S. cerevisiae* is mediated by a group of GATA-type transcription factors. These include Fep1 from *Schizosaccharomyces pombe*, SREA from *Aspergillus nidulans*, SRE from *Neurospora crassa*, SreP from *Penicillium chrysogenum*, and Urbs1 from *Ustilago maydis* (60, 61, 107, 147, 171). These factors regulate, or are predicted to regulate, the expression of genes involved in siderophore production, siderophore transport, and free iron transport (Table 1). GATA factors are a group of transcription factors that are characterized by conserved zinc finger motifs and their ability to bind to a core 5′-GATA-3′ element. The fungal iron-responsive GATA factors contain two zinc finger Cys-X_2-Cys-X_{17}-Cys-X_2-Cys motifs that flank a region that contains four conserved cysteine residues. Adjacent to the C-terminal zinc finger of each factor is a basic region that is conserved in other eukaryotic GATA factors (Fig. 2A). The iron-responsive GATA-type factors repress the transcription of their target genes in response to high iron. Therefore, although the iron-responsive GATA factors are transcriptional repressors and the Aft factors are transcriptional activators, both classes of factors ensure that their target genes are induced when the relevant organism senses that iron is limiting.

The phenotypes of strains that lack an iron-responsive GATA factor are consistent with deregulation of iron metabolism. Wild-type siderophore production is repressed under high iron conditions but derepressed in strains that lack Urbs1, SREA, and SRE (61, 147, 170). Uptake of ^{59}Fe(III) is higher in an *sreA* null strain than in a wild-type strain, and this confers sensitivity to the iron-dependent antibiotics phleomycin and streptonigrin (61). A *fep1* null strain exhibits constitutive cell surface metalloreductase activity and is also sensitive to phleomycin (107). Consistent with these phenotypes, mutant strains that lack the relevant GATA factor exhibit constitutive expression of genes involved in the acquisition of iron from the environment (2, 103, 107, 108, 161). Fep1-dependent repression also requires Tup11 and Tup12 that may act as corepressors in a complex with Fep1 (108). A similar role in the regulation of the iron regulon in *Candida albicans* has also been proposed for the homologous Tup1, although the factor that recruits it to the relevant promoters has not been identified (80).

The number of functional 5′-GATA-3′ elements differs in the target promoters of the iron-responsive GATA factors. Fep1 regulates gene expression via two adjacent sites or a single site depending on the gene in question (107, 108). The full Urbs1-dependent regulation of a gene involved in siderophore synthesis requires two adjacent 5′-GATA-3′ elements, although these are not in themselves sufficient to confer repression of a reporter gene. Therefore, Urbs1 may interact

TABLE 1. Genes that are regulated by metal-responsive transcription factors[a]

Transcription factor[b]	Description	Gene name(s)	Reference(s)
Aft1	Transporters	***FET4***, ***FET5***, ***FTR1***, ***FTH1***, ***SMF3***, ***MRS4***, ***CCC2***, *COT1*	72, 115, 124, 125, 149, 156
	Cu chaperone	***ATX1***	93
	Ferroxidase	***FET3***, ***FET5***	124, 154
	Metalloreductases	***FRE1***, ***FRE2***, ***FRE3***, ***FRE4***, ***FRE5***, ***FRE6***	98, 154
	Cell wall proteins	***FIT1***, ***FIT2***, ***FIT3***	117
	Siderophore transport	***ARN1***, ***ARN2***, ***ARN3***, ***ARN4***	162
	Fe-S biosynthesis	***ISU1***, ***ISU2***	47
	Other[d]	***TIS11***, ***HMX1***, *AKR1*, *PCL5*, *YOR387c*, *YHL035c*, *YMR034c*, *ICY2*, *PRY1*, *YDL124w*	44, 118, 125, 135
Aft2	Transporters	***SMF3***, ***MRS4***, *FTR1*, *COT1*	115, 125
	Cu chaperone	***ATX1***	14
	Ferroxidase	***FET3***, ***FET5***	14, 124
	Metalloreductase	*FRE1*	125
	Cell wall protein	***FIT1***, ***FIT3***, *FIT2*	124, 125
	Fe-S biosynthesis	*ISU1*	125
	Other[d]	*BNA2*, *ECM4*, *LAP4*, *TIS11*, *YOL083w*, *YGR146c*, *YHL035c*	125
Fep1	Transporter	***fip1***$^+$	107
	Ferroxidase	***fio***$^+$	107
	Siderophore transport	***str1***$^+$, ***str2***$^+$, ***str3***$^+$	108
SREA	Siderophore biosynthesis	***sidA***, ***sidB***, ***sidC***, ***amcA***, ***atrH***, ***estA***	103
	Siderophore transport	***mirA***, ***mirB***, ***mirC***	103
	Other[d]	***cycA***, ***acoA***, ***lysF***	102
Urbs1	Siderophore biosynthesis	***sid1***, ***sid2***	2, 161
Ace1	Cu metallothioneins	***CUP1***, ***CRS5***	29, 139, 150
	Cellular stress response	***SOD1***	55
Amt1	Cu metallothioneins	***MT-I***, ***MT-IIa***, ***MT-IIb***	141, 172
	Gene regulation	***AMT1***	174
Crf1	Cu metallothioneins	***MTPI***, ***MTPI1***	46
Mac1	Cu transporters	***CTR1***, ***CTR3***	85, 155
	Metalloreductases	***FRE1***, ***FRE7***	49, 98
	Other[d]	*YFR055w*, *YJL217w*, *YLR213c*	57
Cuf1	Cu transporters	***ctr4***$^+$, ***ctr5***$^+$, ***ctr6***$^+$	7, 9, 84
	Fe transport	***fip***$^{+c}$	84
	Metalloreductase	***frp1***$^{+c}$	84
	Multicopper oxidase	***fio1***$^{+c}$	84
GRISEA	Cu transport	***PaCTR3***	16
	Cellular stress response	***PaSOD2***	18
Crr1	Heme biosynthesis	***CPX1***	68
	Photosystem I maintanence	***CRD1***, ***CTH1***[c]	100, 101
	Electron transfer	***CYC6***	67, 99
Zap1	Zn transporters	***ZRT1***, ***ZRT2***, ***ZRT3***, ***ZRC1***, ***FET4***, ***ZRG17***	94, 95, 96, 149, 159, 166, 167
	Gene regulation	***ZAP1***, *NRG2*	94, 168
	Phosphate/lipid metabolism	***YOL002c***	94
	Metabolic enzymes	***DPP1***, ***ADH4***, *MNT2*, *ADE17*, *TKL2*, *URA10*	94
	Vacuolar proteases	*PRC1*, *PEP4*	94
	Other[d]	***MCD4***, ***ZPS1***, *RAD27*, *ZIP1*, *GRE2*, *BAG7*, *FLO1*, *YNL254c*, *YLL020c*, *YGL258w*, *YOR387c*, *YJR061w*, *YMR086w*, *YOL131w*, *GPG1*, *COS1*, *COS2*, *COS3*, *COS4*, *COS6*, *COS8*, *YJL132w*, *ICY2*, *PST1*, *YBL048w*, *YBL049w*, *YNL234w*, *YDR492w*, *YKL174c*, *PHM7*	94, 159
Mammalian MTF-1	Zn transporters	***ZNT-1***, *ZTL1*	27, 87
	Metallothioneins	***MT-I***, ***MT-II***	66
	Cellular stress response	***AFP***, ***LCN1***, ***PIGF***, ***γ-GCS***	56, 59, 92
	Other	*AHSG*, *CBG*, *PMP22*, *XIST*, *ACVR2b*	92
Drosophila MTF-1	Metallothioneins	***MTNA***, ***MTNB***, ***MTNC***, ***MTND***	38, 163

[a] The genes that are listed are (i) metal regulated and (ii) have a consensus sequence or sequence that resembles the consensus binding site for the relevant transcription factor within the promoter region and/or encode for a protein that is involved in metal metabolism. Genes that have been analyzed in such a way to demonstrate a more direct interaction with the factor in question are shown in bold. Examples of experimental evidence that is consistent with a direct interaction include analysis of expression in the presence and absence of the wild-type or constitutive alleles of the relevant factor and/or the use of reporter constructs containing the metal-responsive element(s) of the target gene in question.

[b] No target gene has been identified for the SRE and SreP transcription factors.

[c] Genes that are repressed, rather than activated, by the named transcription factor.

[d] Genes which do not fall into any of the listed categories.

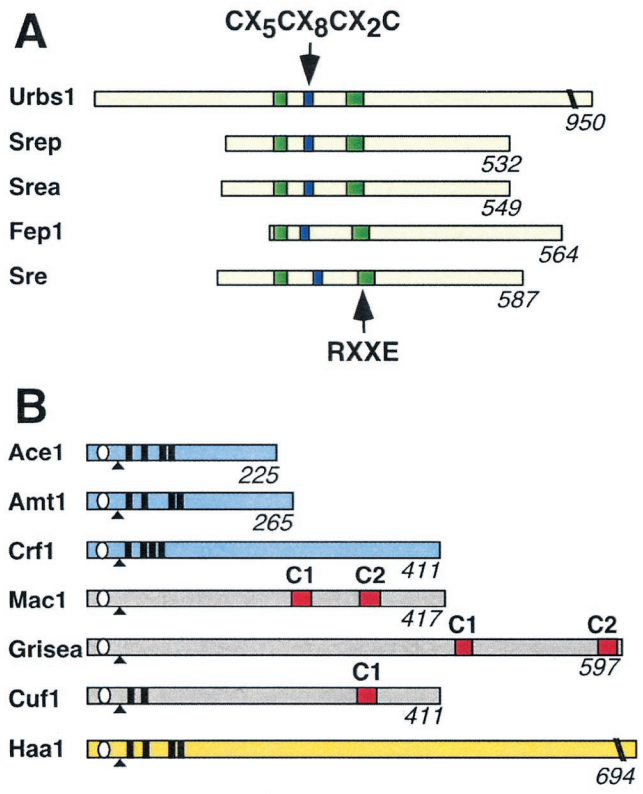

FIG. 2. Schematic representation of the fungal iron-responsive GATA factors (A) and copper-responsive transcription factors (B). Shown are Mac1, Ace1, and Haa1 from *S. cerevisiae*, Fep1 and Cuf1 from *S. pombe*, SREA from *A. nidulans*, SRE from *N. crassa*, SreP from *P. chrysogenum*, Urbs1 from *U. maydis*, GRISEA from *P. anserina*, Amt1 from *C. glabrata*, and Crf1 from *Y. lipolytica.* In panel A, the conserved zinc finger motifs (green rectangles), the cysteine-rich region (blue rectangle), and the RXXE motif of the iron-responsive GATA factors are indicated. In panel B, the following motifs are shown: conserved zinc modules (white ovals), conserved (R/K)GRP motifs (black triangles), positions of N-terminal Cys-X-Cys or Cys-X_2-Cys motifs outside the zinc motif (black rectangles), and positions of the C1 and C2 motifs (red rectangles). Proteins that are active under copper-limiting or copper-replete conditions are shown in grey and blue, respectively. Proteins that are not copper-responsive are shown in yellow.

with other GATA sites in this particular promoter region (2). The in vitro affinity of SRE for two adjacent 5′-GATA-3′ elements is dependent on the spacing between those two sites (62). Urbs1 and Fep1 have a higher in vitro affinity for one site when the target DNA contains two adjacent 5′-GATA-3′ elements. The loss of this high-affinity site results in a greater loss of in vivo repression of a reporter gene than does loss of the low-affinity site (2, 107). In addition, the phenotypes of various Urbs1 mutants suggest that, of the two zinc fingers, the C-terminal finger is more important for DNA binding (3).

Evidence to date suggests that the iron-responsive GATA factors bind iron. Recombinant SRE is reddish brown in color and gives a spectrum that is characteristic of iron-binding proteins, which is lost when the protein is reduced (62). The diagnostic spectrum of the wild-type protein is lost with proteins that contain cysteine-to-serine mutations in the conserved region between the zinc fingers. In vivo, these substitutions result in a constitutive repressor that does not respond to iron (62). Furthermore, the in vitro DNA-binding ability and stability of recombinant Fep1 is dependent on the protein being expressed in cells that are grown in high-iron medium before the protein is purified (107). In addition to the conserved cysteines between the zinc fingers, it has also been proposed that a conserved RXXE motif in the C-terminal zinc finger is a potential iron-binding site (107). An arginine-to-leucine mutation in the same motif in Urbs1 renders it unable to respond to iron (3). As with the iron-responsive factors from *S. cerevisiae*, further work is required to determine the precise mechanism of iron sensing by these factors.

COPPER

A number of homeostatic mechanisms have been identified which ensure that copper is maintained at a level sufficient for, but not toxic to, cell growth. In mammals, posttranslational mechanisms, such as the intracellular trafficking of copper transporters and the copper-stimulated endocytosis and degradation of proteins involved in copper uptake, play a major role in copper homeostasis (109, 112, 113). Although posttranslational control of transporters exists in fungi, copper homeostasis in these organisms is also mediated by the transcriptional regulation of genes involved in copper acquisition, mobilization, and sequestration (105). To date, six copper-responsive fungal factors have been characterized in detail. Ace1 (also known as Cup2), Amt1, and Crf1 activate gene expression in response to elevated copper while Mac1, GRISEA, and Cuf1 activate gene expression in response to copper deficiency. Homeostasis through copper-responsive transcriptional regulation has been observed in insects and plants, as well as in fungi, suggesting that this mechanism of copper control is widespread in nature (69, 126, 169).

Factors that are activated in response to copper regulate the expression of genes encoding proteins involved in copper sequestration and/or protection against copper toxicity (Table 1). In *S. cerevisiae*, resistance to copper is primarily mediated by the Ace1-dependent induction of the *CUP1* gene (Fig. 1B) (139, 150). *CUP1* encodes a small, cysteine-rich, copper-binding metallothionein that protects cells by sequestering copper and thereby preventing its toxicity (23, 42, 77, 153). Ace1 also regulates the expression of a second metallothionein gene (*CRS5*) and the copper and zinc superoxide dismutase gene (*SOD1*) (29, 55). Functional orthologs of Ace1 confer copper resistance in *Candida glabrata* (Amt1) and *Yarrowia lipolytica* (Crf1) (46, 173). Although Amt1 protects cells from copper by regulating the expression of three metallothionein genes, metallothionein expression is still copper responsive in a *crf1* mutant strain (46, 141, 172). This latter result suggests that Crf1 guards against copper overload by regulating the expression of a yet-unidentified target gene(s) (46).

Regulatory factors that are active during copper deficiency regulate the expression of genes encoding proteins involved in increasing cytosolic copper (Table 1). In *S. cerevisiae*, Mac1 protects cells from copper starvation by activating the expression of the high-affinity copper uptake systems encoded by *CTR1* and *CTR3* (Fig. 1A) (85, 155). Mac1 also regulates the expression of a cell surface Fe^{3+}/Cu^{2+} reductase (*FRE1*) and

a putative reductase of unknown cellular localization (*FRE7*) (49, 98). The posttranslational degradation of Ctr1 under conditions of excess copper also requires Mac1. It is possible that an uncharacterized Mac1 target gene (Table 1) encodes a protein that is essential for this regulation or that Mac1 itself functions as a protease or protease-recruiting factor under copper-replete conditions (158).

High-affinity copper uptake is regulated at the transcriptional level in *S. pombe* and *Podospora anserina* by the factors Cuf1 and GRISEA, respectively (7, 16, 84). In addition to directly regulating copper uptake, Cuf1 stimulates mobilization of copper from vacuolar stores by regulating the expression of the *CTR6* vacuolar efflux system (9). GRISEA activates the expression of *P. anserina SOD2*, a gene that is not copper regulated in *S. pombe* or *S. cerevisiae* (17). *P. anserina SOD2* encodes mitochondrial manganese superoxide dismutase. In *P. anserina*, low intracellular copper levels lead to reduced activity of the copper-requiring enzyme, cytochrome *c* oxidase. This in turn results in the induction of an iron-dependent pathway that utilizes an alternative terminal oxidase. Induction of *P. anserina SOD2* under copper-limiting conditions may therefore be important for protection from oxidative stress inherent in the utilization of the alternate oxidase (16–18).

In addition to activating copper transporter genes, Cuf1 represses the expression of the iron-regulated *fip1*$^+$, *fio1*$^+$, and *frp1*$^+$ genes that encode proteins required for iron uptake (84). Similar to *S. cerevisiae*, iron uptake in *S. pombe* requires copper. Cuf1-dependent repression of these genes therefore ensures that iron uptake is inhibited under copper-limiting conditions. As copper levels increase (through Cuf1-dependent copper uptake and copper mobilization), Cuf1 is inactivated, which leads to the loss of Cuf1-mediated repression of *fip1*$^+$, *fio1*$^+$, and *frp1*$^+$ (84). It is currently unknown whether Cuf1 mediates this repression by recruiting a corepressor to these promoters or whether Cuf1 inhibits binding of a transcriptional activator. A similar regulatory pathway may also exist in *S. cerevisiae*, since the iron-regulated *FET3* gene also appears to show Mac1-dependent repression (84). However, it is not clear whether this is a direct result of Mac1 acting as a repressor at the *FET3* promoter or whether this is a pleiotropic affect of altered iron homeostasis in strains that lack or express a constitutive allele of *MAC1*. Thus, the ability of Cuf1, and possibly Mac1, to act as both a repressor and activator allows the coordinated expression of genes involved in both copper and iron homeostasis.

A number of structural domains are conserved between the six known copper regulatory factors (Fig. 2B). Ace1, Amt1, and Crf1 all contain a zinc-binding domain, a conserved (R/K)GRP sequence motif, and eight cysteine residues that are arranged in Cys-X-Cys or Cys-X_2-Cys motifs (45, 137, 142). The cysteine-rich motifs form a polycopper cluster that binds 4 Cu(I) ions cooperatively while the zinc-binding domain and (R/K)GRP motif are essential for minor groove site-specific binding (32, 41, 53, 81, 142, 143). Mac1, GRISEA, and Cuf1 share regions of homology to Ace1 but lack all the cysteine-rich motifs required in forming the Ace1-Amt1 N-terminal polycopper cluster. The C terminus of Mac1 contains two Cys-X-Cys-X_4-Cys-X-Cys-X_2-Cys-X_2-His motifs that have been designated C1 and C2 (or REPI and REPII, respectively) (Fig. 2B) (54, 78, 175). The C1 and C2 motifs lie within transactivation domains and bind four Cu(I) ions in a polycopper cluster (20, 73). Similar to Mac1, GRISEA contains two cysteine-rich domains (15). Cuf1 contains only one of these motifs, designated C1 (84).

An important facet of copper homeostasis is that copper regulates the activity of each transcription factor. Evidence to date indicates that the conserved structural domains within each class of copper regulatory factors are important for copper sensing. Copper-dependent DNA-binding activity primarily regulates Ace1 and Amt1 activity. Both factors bind as a monomer, in a copper-dependent manner, to upstream activating sequences (22, 45, 70, 140, 172). The copper-induced gene activation is thought to be mediated by the formation of the N-terminal polycopper cluster in response to copper, which converts Ace1 and Amt1 from a nonactive form to an active DNA-binding form (reviewed in reference 152). Haa1, a transcriptional activator in *S. cerevisiae*, contains the eight conserved cysteine residues that are required for polycopper cluster formation in Ace1, yet is not regulated by copper. Amino acids present in the Haa1 N-terminal region but not present in Ace1 or Amt1 may disrupt polycopper cluster formation and prevent this domain from being used as a copper regulatory domain (79). Additional regulatory mechanisms control both Amt1 and Crf1 activity. Amt1 autoregulates its own expression, a critical factor in copper resistance, since cells that are unable to autoactivate *AMT1* are sensitive to growth on copper (174). Crf1 activity is subject to copper-dependent nuclear translocation (46).

Mac1 mediates the response to copper limitation by binding as a homodimer, in a site-specific manner, to copper-responsive *cis*-acting elements (CuREs) that are located in the promoter regions of target genes (71, 76, 85, 131, 155). In vivo, activation of gene expression requires multiple CuREs, which are arranged in tandem or as inverted repeats and have a synergistic effect on gene expression (74, 85, 155). On exposure to copper, repression of Mac1 is primarily mediated by a copper-dependent interaction between the C1 domain and the DNA-binding domain. This interaction inhibits both transactivation domain function and DNA-binding activity (54, 74, 85). In support of this model, mutations that substitute single cysteine residues in the Mac1 C1 motif lead to a total loss of copper-responsive regulation (78, 155, 175).

Cuf1 activity is similarly controlled by a copper-dependent interaction between the DNA-binding domain and the C1 domain (8). However, a number of differences between Mac1 and Cuf1 regulation have been observed. The N-terminal region of Cuf1 exhibits a higher percentage of sequence identity with the corresponding Ace1 domain than with the N-terminal domain of its functional homolog Mac1. Indeed, Cuf1 can activate Ace1 target gene expression when introduced into an *ace1* null *S. cerevisiae* strain (7, 8). A second difference between Mac1 and Cuf1 is that the substitution of cysteine residues in the Cuf1 C1 domain only leads to a partial loss of regulation. This result suggests that the cysteine residues of Cuf1 are not equally involved in copper coordination (8). Similarities and differences between the regulation of Mac1 and GRISEA by copper are also observed. For example, copper-responsive repression of GRISEA is mediated by an interaction between the DNA-binding domain and the C2 domain rather than the C1 domain (15).

While the copper-dependent control of Mac1 activity via an intramolecular interaction remains undisputed, more-recent studies of Mac1 have superimposed a number of additional regulatory mechanisms. Mac1 must be phosphorylated to bind to CuREs, and homodimerization of Mac1 is essential for maximal in vivo activity (65, 76, 131). It is noteworthy that overexpression of Mac1 constructs that lack the homodimerization domain are fully functional proteins (74, 148). Homodimerization of Mac1 may therefore allow a protein that is normally expressed at a low level to achieve maximal activation of target gene expression. A third additional level of regulation is that in the presence of excess copper, C-terminally tagged Mac1 undergoes a rapid copper-dependent degradation. However, this effect is lost when Mac1 is overexpressed (74, 175). Finally, the C2 transactivation domain may have a role in modulating the activity of Mac1 (78, 148). Further studies that identify other proteins required for Mac1 regulation may reveal the finer details of how Mac1 responds to copper.

In addition to Mac1 and its functional homologs, novel copper regulatory factors may also exist in the plant kingdom. Copper is an essential cofactor of a number of the enzymes required for photosynthesis. During copper deficiency, the photosynthetic algae *Chlamydomonas reinhardtii* can bypass this copper requirement by decreasing its reliance on copper-requiring enzymes and using alternative enzymes that utilize heme cofactors. At the transcriptional level, this transition is mediated by the Crr1-dependent induction of *CPX1* and *CYC6* (120, 121). These genes encode the enzyme coproporphyrinogen oxidase that is required for heme biosynthesis and cytochrome c_6, a heme-containing electron transfer catalyst. Crr1 also reciprocally regulates the expression of the partially redundant genes *CRD1* and *CTH1*, which are required for the maintenance of photosystem I under copper-limiting and -replete conditions, respectively (100, 101). Although the precise genetic locus of Crr1 has yet to be identified, a number of observations suggest that Crr1 is not simply an ortholog of Mac1. First, one CuRE is both necessary and sufficient to mediate copper-responsive regulation (119). Second, the consensus sequence of GTAC, rather than the Mac1 consensus 5′-TTTGC(T/G)C(A/G)-3′, is found in all known Crr1 target genes and is essential for copper-responsive transcription (119). Finally, Crr1 possibly responds to Cu^{2+} not Cu^{+}, since Hg^{2+} and not Ag^{+} will also repress Crr1 target gene transcription (119).

ZINC

To a major extent, zinc homeostasis generally parallels copper homeostasis in that both posttranslational and transcriptional homeostatic regulatory mechanisms function together to maintain zinc at an optimal level under conditions of either zinc limitation or zinc excess. For example, in *S. cerevisiae*, expression of the high-affinity zinc uptake gene *ZRT1* increases in response to zinc limitation, whereas under zinc-replete conditions, Zrt1 undergoes zinc-induced endocytosis and is degraded in the vacuole (39). However, unlike iron and copper, zinc-responsive transcription factors are found in fungi, mammals, fish, and possibly plants, suggesting that the transcriptional control of genes involved in zinc homeostasis is of universal importance (31, 58, 110, 151, 168). To date, two factors that control gene expression in response to zinc have been characterized in detail. These are Zap1 from *S. cerevisiae*, which activates gene expression in response to zinc deficiency, and mammalian MTF-1, which is activated by zinc (151, 168).

Under zinc-limiting conditions, Zap1 increases the expression of three zinc uptake systems encoded by the *ZRT1*, *ZRT2*, and *FET4* genes (Fig. 1A) (149, 166, 167). Zap1 also stimulates the release of zinc from the vacuolar zinc store by activating the expression of the *ZRT3* vacuolar efflux system (95). A fifth target of Zap1 is *ZRC1*, a gene that encodes a vacuolar zinc influx system (96). Although it seems counterintuitive that Zap1 up-regulates the expression of a gene associated with lowering cytoplasmic zinc levels, recent studies have revealed that the increased expression of *ZRC1* in response to zinc limitation is a proactive mechanism to protect zinc-limited cells from possible exposure to high zinc levels (97). In addition to the five zinc transporter genes, Zap1 regulates the expression of 42 other genes, some of which may have additional roles in zinc homeostasis (Table 1) (94).

In mammals, MTF-1 plays a central role in protecting cells against zinc toxicity. This is partly achieved by increasing the expression of *MT-1* and *MT-2*, two genes that encode zinc-binding metallothioneins (66). MTF-1 also lowers cytoplasmic zinc levels by regulating the expression of a zinc efflux system encoded by the *ZnT-1* gene (87). A further putative target gene of MTF-1 is *hZTL1*, a gene that encodes a zinc uptake transporter that is localized to the enterocyte apical membrane (27). While increased *hZTL1* expression may assist efficient uptake of zinc from a zinc-rich diet, this apparent regulation counteracts other homeostatic mechanisms. Perhaps this unusual regulation ensures that zinc is effectively absorbed from the intestine while other transcriptional and posttranslational homeostatic mechanisms maintain cellular zinc at an optimal level under these conditions.

In addition to regulating genes involved in zinc homeostasis, MTF-1 regulates the expression of a number of other genes (Table 1) (92). In mice, *MTF-1* is an essential gene, with knockout mice dying in utero at approximately day 14 of gestation due to degeneration of hepatocytes (59). Although the exact reason for the lethality of the *MTF-1* knockout is currently unknown, a number of candidate target genes of MTF-1 that are essential for embryonic development (C/EBPα and α-fetoprotein) could provide clues to the lethality phenotype (92). Contrary to the lethality of *MTF-1* in mice, an *MTF-1* knockout in *Drosophila melanogaster* is viable (38). In *Drosophila*, however, copper is a more potent inducer of MTF-1 activity than zinc and MTF-1 plays a dual role in regulating genes involved in resistance to copper toxicity and in preventing copper deficiency (38, 163). Thus, MTF-1 can have species-specific cellular roles in addition to zinc homeostasis.

Zap1 and MTF-1 have a number of features that are common to transcriptional activators, which include transactivation domains and DNA-binding domains containing C_2H_2-type zinc finger motifs (Fig. 3A). Zap1 contains two acidic activation domains (168). The first activation domain is located at the N terminus in a region rich in cysteine and histidine residues, and the second activation domain maps to a region containing two C_2H_2-type zinc finger domains (11). A further five C-terminal zinc finger domains are all essential for Zap1 DNA-binding activity (10, 40, 165). *MTF-1* encodes a 72.5-kDa protein that

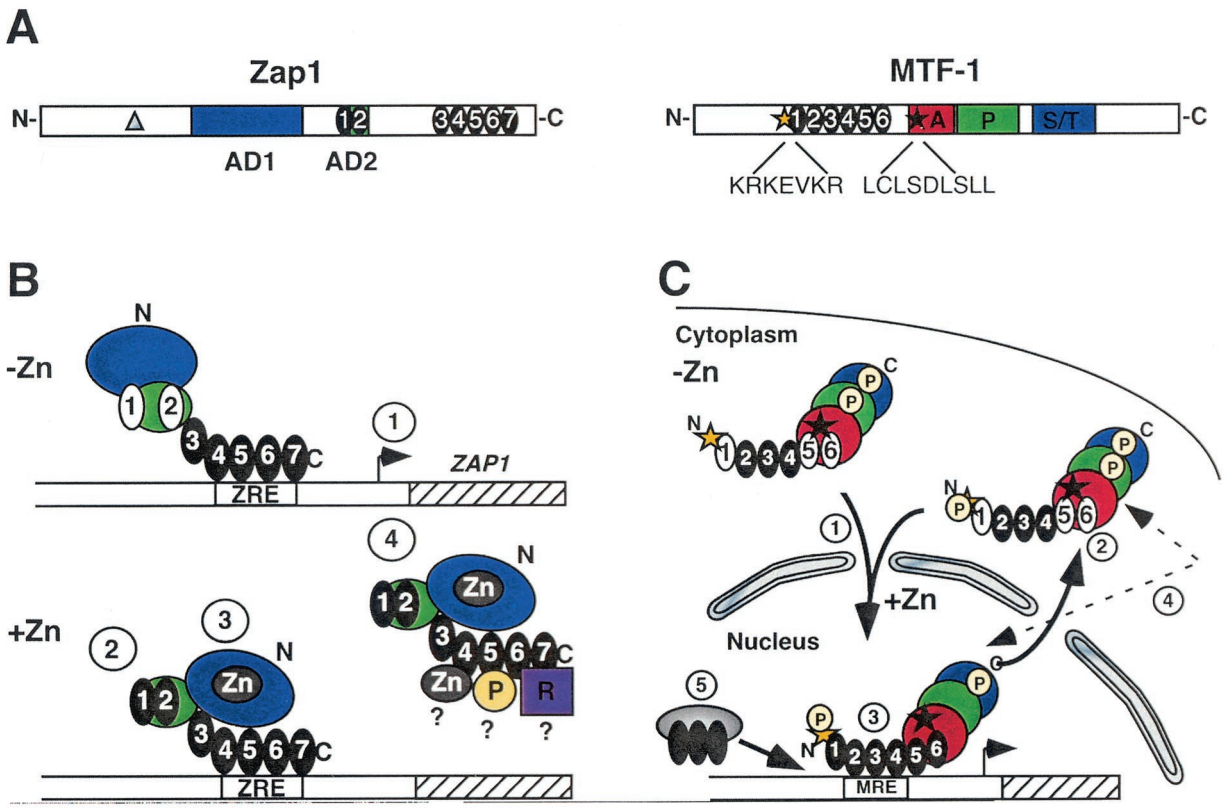

FIG. 3. (A) Schematic diagram of *S. cerevisiae* Zap1 and mouse MTF-1. The following are shown: zinc finger domains (numbered black ovals), position of the cysteine-to-serine mutation in Zap1-1^{up} (grey triangle), MTF-1 nuclear localization signal (gold star), MTF-1 NES (black star), and MTF-1 acid-, proline-, and serine/threonine-rich activation domains (red, green, and blue boxes labeled A, P, and S/T, respectively). (B) The multiple levels of Zap1 regulation: 1, autoregulation; 2, AD2 repression; 3, AD1 repression; 4, DNA-binding control. (C) The multiple levels of MTF-1 regulation: 1, nuclear translocation; 2, nucleocytoplasmic shuttling; 3, DNA-binding control; 4, posttranslational modification by phosphorylation; 5, interactions and binding inhibition by other factors. The absence of zinc in a metalloregulatory finger is indicated by a white numbered oval. Possible phosphorylation events (yellow circle labeled P), a putative repressor (purple square), and target genes (hatched boxes) are shown.

contains six C_2H_2-type zinc finger domains and three transactivation domains (Fig. 3A) (21, 122).

A critical feature in understanding zinc homeostasis is determining how these factors sense zinc. Multiple regulatory mechanisms contribute to the inactivation of Zap1 by zinc (Fig. 3B). At the transcriptional level, Zap1 binds to a zinc-responsive element located within its own promoter and autoregulates its own expression. Zap1 activity is also regulated by up to three posttranslational mechanisms (12, 165, 168). The most understood regulatory mechanism is the autonomous repression of activation domain 2 (AD2) by zinc. Both of the zinc finger domains that are located in AD2 (Znf1 and Znf2) are required for zinc-responsive repression. In vitro, zinc binds with a slightly lower affinity and, notably, with a much higher lability to the Znf1-Znf2 pair relative to a control pair of zinc finger domains (11). Moreover, in vivo, residues that form the packing interface between the two fingers are an essential component of zinc-responsive repression. Thus, as zinc increases, the zinc occupancy of Znf1 and Znf2 may result in an interfinger, protein-protein interaction that masks critical residues that are essential for transactivation domain function (11).

In the absence of AD2 regulation, both AD1 and the Zap1 DNA-binding domain are negatively regulated by zinc by independent mechanisms (12). Repression of AD1 most likely involves a zinc-dependent intramolecular interaction with the Zap1 DNA-binding domain that masks activation domain function. In support of this model, AD1 is rich in potential zinc-coordinating ligands and repression of AD1 by zinc requires the Zap1 DNA-binding domain. In addition, a less-zinc-responsive mutant allele of *ZAP1* (*ZAP1-1^{up}*) encodes a cysteine-to-serine mutation in a region that is immediately upstream from AD1 (Fig. 3A) (12, 168). Zap1 DNA-binding activity may also be regulated by zinc, since the Zap1 DNA-binding domain is able to confer zinc-responsiveness onto a heterologous activation domain (12). At present, the precise mechanism by which zinc inhibits Zap1 DNA-binding activity is unknown.

Unlike Zap1, which is primarily regulated by zinc, MTF-1 activity can be regulated by zinc, other divalent metal ions, and various stress conditions in vivo (4, 51, 91). In vitro, zinc stimulates transcriptional induction by MTF-1, whereas induction in response to cadmium, copper, or H_2O_2 additionally requires the presence of zinc-saturated metallothionein (164). More-

over, in *Drosophila*, MTF-1 is primarily regulated by copper, whereas in transfected mammalian cells, *Drosophila* MTF-1 responds to zinc like mammalian MTF-1 (163). Thus, other aspects of metal ion homeostasis, such as metal release from metallothionein, may influence the primary metal specificity of MTF-1.

MTF-1 is regulated at multiple levels by zinc (Fig. 3C) (4, 51, 91). The first level of regulation of MTF-1 involves its cellular localization. Under noninducing conditions, MTF-1 predominantly resides in the cytoplasm. Upon addition of zinc or cadmium, MTF-1 rapidly translocates to the nucleus (128, 133). Mutation of the MTF-1 NES results in the retention of MTF-1 in the nucleus in a form that is able to bind to a metal response element (MRE) in response to zinc but is unable to activate transcription. Thus, a second level of regulation involving the MTF-1 cellular position could potentially be nucleocytoplasmic shuttling, i.e., MTF-1 might undergo a constant cycle of activation, nuclear import, deactivation, nuclear export, and then reactivation (128). The third level of regulation is the control of DNA-binding activity (13, 30, 66, 82). MTF-1 DNA-binding activity increases upon the addition of zinc. Consequently, one model is that one or more of the zinc finger domains bind zinc with a low affinity or low stability; thus, full DNA-binding activity is only achieved upon full metallation of the regulatory zinc finger(s) (66, 151).

Recent in vitro studies have clearly demonstrated that there is conformational heterogeneity in the MTF-1 DNA-binding zinc fingers such that the zinc bound to Znf5, Znf6, and to a lesser extent, Znf1 is less stable than the zinc bound to the remaining finger domains (Znf2, Znf3, and Znf4) (25, 26, 52). While Znf2, Znf3, and Znf4 form the core DNA-binding domain in vitro, deletion of Znf1 or Znf5 and Znf6 leads to attenuation of zinc-induced MTF-1 DNA binding at the endogenous MT-1 promoter in vivo (75). Thus, Znf1, Znf5, and Znf6 are required for maximal binding under zinc-replete conditions (75). Similar effects are not seen in mutants containing deletions of Znf5 or Znf6, whether examined in vitro or with an MRE-reporter construct, suggesting that binding zinc in these regulatory fingers may stabilize an MTF-1–chromatin complex (13, 75, 82).

MTF-1 activity is also controlled by phosphorylation. MTF-1 is phosphorylated in both an uninduced and an induced state. However, the level of phosphorylation is stimulated two- to fourfold by the addition of zinc. Kinase inhibitor studies have revealed that this phosphorylation is an essential component of zinc-responsive MTF-1 activation. Since kinase inhibitors have little effect on the nuclear import or DNA-binding activity of MTF-1, signal transduction pathways may use phosphorylation or dephosphorylation to control activation domain function (88, 127). Finally, under specific conditions, other factors can influence MTF-1 activity. The precise activity of MTF-1 may also be dependent on the MRE sequence, the chromatin architecture of the target loci, cell type, developmental stage of growth, and growth conditions (1, 5, 50, 104).

Both Zap1 and MTF-1 use regulatory zinc finger domains to sense zinc; however, it is currently unknown what properties of a zinc finger make it regulatory. The regulatory zinc fingers all match the consensus zinc finger sequence (Phe/Tyr-X-Cys-X_{2-4}-Cys-X_3-[Phe]-X_5-Leu-X_2-His-X_{2-3}-His), with the exception of the conserved central phenylalanine. In Znf1 and Znf2 from Zap1, this residue is a cysteine and glycine, respectively. Attempts to convert the Zap1, Znf1, and Znf2 fingers back to the consensus sequence (by converting the cysteine and glycine residues at the finger tip to phenylalanine) have no effect on AD2 activity, suggesting that the high lability of the zinc bound to these fingers is not simply caused by these substitutions (11). Importantly, these zinc fingers function together as a pair, suggesting that other residues that stabilize pair formation may have important regulatory functions. In MTF-1, Znf5 fluctuates between the canonical $\beta\beta\alpha$ structure and another structure or structures upon addition of excess zinc (52). Znf5 contains five additional potential zinc-coordinating ligands that are located in the Cys-X_4-Cys loop and the α-helix. The high conservation of these residues and the properties of Znf5 have led to the speculation that these residues may bind an additional zinc ion that destabilizes Znf5 (52). Thus, a precise knowledge of what makes these particular fingers bind zinc with a higher lability than other fingers will help us to understand their regulatory function.

Another unanswered question is what zinc pools do MTF-1 and Zap1 sense. In *Escherichia coli*, the zinc sensors Zur and ZntR respond to femtomolar levels of zinc (less than 1 atom of zinc per cell) (106). These data suggest that the majority of cellular zinc is bound in a yet-unidentified bioavailable zinc pool that could consist of proteins, small molecules, or other macromolecules that either strongly or weakly chelate zinc. Because of the predicted low levels of free zinc, it has been proposed that zinc-trafficking factors may be required to deliver zinc to proteins (106). Although the precise level of free zinc in eukaryotic cells is unknown, it is interesting that both MTF-1 and Zap1 sense zinc in the nanomolar to subnanomolar range (11, 52). If free intracellular zinc levels are maintained at much less than nanomolar concentrations in eukaryotic cells, then the eukaryotic zinc sensors must be detecting fluctuations in a bioavailable zinc pool. Studies with eukaryotic sensors may therefore help us to answer questions such as what molecule(s) or protein(s) potentially delivers zinc to Zap1 and MTF-1. These studies also raise many other interesting questions, such as whether all zinc-sensing factors rely on regulatory zinc fingers or whether other types of zinc domains, such as the GATA or LIM domain, can be used.

CONCLUSIONS

The transcription factors described here have provided insight into the molecular mechanisms involved in the control of eukaryotic metal ion homeostasis. The regulation of these proteins at multiple levels is a recurring theme and may be characteristic of all eukaryotic metalloregulatory factors. There is also compelling evidence that these factors directly sense metal, although, to date, there is no direct in vivo evidence that their metal occupancy changes with cellular metal ion status. Therefore, alternative mechanisms of sensing, such as signal transduction pathways, that involve protein kinases cannot be discounted at present.

It is likely that other eukaryotic metal-responsive transcription factors exist. There are many fungal genes that are regulated in response to metal ions independent of the factors described in this review (19, 90, 102). In other eukaryotes, metal-regulated genes are continuously being identified, sug-

gesting that other metalloregulators exist (for examples, see references 37, 58, 69, 86, 110, 111, 126, and 132). The influence of metals on human health has generated considerable interest in the understanding of the principles involved in metal homeostasis. As more metal-responsive transcription factors are discovered in both plants and mammals, it should become apparent whether or not there are general principles of metal ion sensing that are conserved throughout the eukaryotes. This will be indispensable to our understanding of intracellular metal homeostasis.

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